II.D.2 Biological Systems for Hydrogen Photoproduction

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Objectives

- Generate algal hydrogenase mutants with higher O_2 tolerance to function with aerobic H_2 production systems being developed in collaboration with Oak Ridge National Laboratory (ORNL) and University of California (UC) Berkeley.
- Optimize H_2 photoproduction using an algal production system discovered by UC Berkeley and NREL.
- Generate a recombinant cyanobacterial system for H_2 photoproduction using an O_2-tolerant hydrogenase from the photosynthetic bacterium, *Rubrivivax gelatinosus* CBS.

Technical Barriers

This project addresses the following technical barrier from the Hydrogen Production section of the Hydrogen, Fuel Cells and Infrastructure Technologies Program Multi-Year Research, Development and Demonstration Plan:

- K. Continuity of Photoproduction

Approach

Subtask 1 (Molecular Engineering of the Algal Hydrogenase):

- Perform computational simulations to identify *in silico* mutations that inhibit O_2 (but not H_2) access to/from the catalytic site; apply site-directed mutagenesis techniques to generate the mutants identified above and test them for O_2 tolerance.
- Use *in vitro* random mutagenesis to generate additional hydrogenase mutants; develop a high-throughput assay to screen positive transformants for O_2 tolerance.
- Genetically combine an optimal O_2-tolerance phenotype with truncated antenna (UC Berkeley) and proton channel (ORNL) mutants.

Subtask 2 (Biochemical and Process Engineering):

- Further optimize a sulfur-deprived system for H_2 production using either cell suspensions or immobilized algal cultures.
- Demonstrate H_2 production at an average rate of 2 ml/(L x h) by 2010.

Subtask 3 (Recombinant Cyanobacterial H_2 Production):

- Transfer the CBS O_2-tolerant hydrogenase genes into a *Synechocystis sp*. cyanobacterial host and test for O_2 tolerance.

- Link cyanobacterial photosynthetic water oxidation to CBS-hydrogenase-catalyzed H_2 production *in vivo*.

Accomplishments

- Performed molecular dynamics modeling of O_2 diffusion in the algal hydrogenase and identified targets for site-directed mutagenesis aimed at preventing O_2 inactivation.
- Discovered a heterologous expression system (*E. coli*) for production of large amounts of active algal hydrogenase.
- Optimized light intensity, sulfate concentration and medium dilution rates to operate the continuous H_2 production system using suspended cultures; extended the duration of H_2 production from 240 hours to a total of 6 months.
- Demonstrated a 1.7 times higher rate of H_2 production (per volume of photobioreactor) with fiberglass-immobilized algal cultures.
- Constructed a plasmid to create a hydrogenase knockout in the wild-type (WT) cyanobacterial host.
- Constructed an expression system in *E. coli* to mass produce cyanobacterial ferredoxin to link cyanobacterial photosynthetic water oxidation function to the CBS hydrogenase activity *in vitro*.

Future Directions

Subtask 1 (Molecular Engineering of the Algal Hydrogenase):

- Generate a H_2 gas diffusion simulation model of a hydrogenase and assess the effects of selected O_2-tolerance mutations on H_2 diffusion.
- Continue the current iterative process between computational simulations and experimental site-directed mutagenesis to generate and test additional O_2-tolerant mutants.
- Initiate the random mutagenesis approach.

Subtask 2 (Biochemical and Process Engineering):

- Complete the optimization of the continuous H_2-production system.
- Engineer a + S/-S cyclic H_2 production batch system using algal cultures immobilized on different matrices.

Subtask 3 (Recombinant Cyanobacterial H_2 Production):

- Construct a hydrogenase knockout mutant in *Synechocystis*.
- Link cyanobacterial photosynthesis to the O_2-resistant bacterial hydrogenase *in vitro* and ultimately *in vivo*.

Introduction

Two classes of oxygenic photosynthetic organisms can photoproduce H_2 from water: eukaryotic green algae and prokaryotic cyanobacteria. This capability requires the coordinated operation of the photosynthetic water oxidation machinery (that generates O_2, reductants and protons from water) and the hydrogenase enzyme (that re-combines protons and reductants to produce H_2 gas). The catalytic center of green algal Fe-hydrogenases is composed of a unique 2Fe2S center, while the cyanobacterial hydrogenases are classified as NiFe-hydrogenase due to the presence of Ni, Fe and S in their catalytic centers. Both classes of hydrogenases are sensitive to O_2, a by-product of photosynthetic water oxidation, and O_2 inactivation prevents sustained H_2 production by either organism in the light. The continuity of photoproduction is one of the three technical barriers (Barrier K) identified by the DOE Hydrogen, Fuel Cells and Infrastructure Technologies Program's Multi-Year R,D&D Plan as currently hindering the development of photobiological H_2 production systems.

Our current project addresses three different strategies for surmounting the O_2 sensitivity of photobiological H_2-producing organisms. These

include (a) molecular engineering efforts to alleviate the O_2 sensitivity of the algal hydrogenase (Subtask 1), (b) use of a physiological switch to separate O_2 and H_2 production (Subtask 2), and (c) generation of a recombinant cyanobacterium expressing a previously discovered O_2-tolerant hydrogenase from a CO-utilizing bacterium (new Subtask 3).

Approach

Subtask 1 (Molecular Engineering of the Algal Hydrogenase): Previous work on bacterial NiFe-hydrogenases demonstrated the existence of a hydrophobic channel connecting the surface of the enzyme to its catalytic site (1,2). This observation led us to hypothesize that inactivation of the algal Fe-hydrogenase depends on access of O_2 to the enzyme's catalytic site through a similar channel. The hypothesis was supported by the increased O_2 tolerance of a site-directed mutant containing a bulkier amino acid residue that partially shields the catalytic site and might prevent O_2 access from the surface (King et al., patent pending). This year, we generated a computational simulation model of O_2 gas diffusion through an Fe-hydrogenase enzyme in collaboration with the Beckman Institute of the University of Illinois and NREL's Computational Sciences Center. The initial results indicated that O_2 diffusion occurs mainly through the hydrophobic channel and validated our mutagenesis approach. Future "knowledge-based" work will involve an iterative process between computational simulation and experimental site-directed mutagenesis to identify additional amino acid substitutions that may further improve O_2 tolerance of the enzyme.

Since O_2 sensitivity could possibly be conferred by changes in the hydrogenase protein structure distant from the hydrophobic channel (and not obvious in the gas diffusion simulations), our approach will also include the use of alternative molecular engineering approaches to generate desirable mutants. High-throughput screening assays are currently being developed to select for desired mutants.

Subtask 2 (Biochemical and Process Engineering): A shorter-term approach to circumvent the O_2-sensitivity issue of green algal H_2 production was developed jointly in 2000 by the University of California, Berkeley, and NREL. This work was based on the metabolic shift from O_2 to H_2

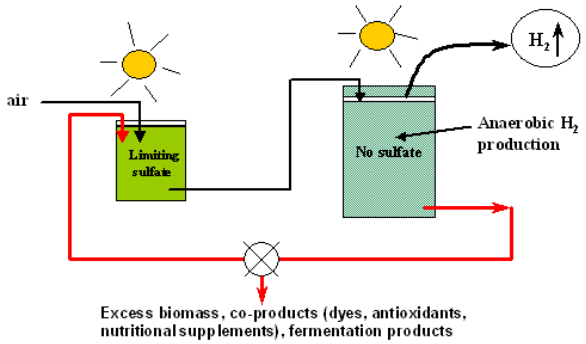

Figure 1. Schematic Diagram of the Two-Photobioreactor, Continuous Algal H_2-Photoproduction System

production, induced by depriving algal cultures of sulfate (3). The original system, which was designed to operate in cycles of +S and –S, was later converted into a continuous H_2-producing system at NREL (Figure 1). This resulted in a decrease by a factor of 3 in the estimated cost of H_2 production (4). Current efforts focus on optimizing the operation of the continuous system.

As an alternative approach, we are assessing the feasibility of immobilizing algal cells at high density onto different classes of matrices in order to facilitate the cycling of the cultures between +S and –S conditions.

Subtask 3 (Recombinant Cyanobacterial H_2 Production): A complementary strategy to overcome the O_2-sensitivity problem in algae is to construct a recombinant cyanobacterial system in which the O_2-sensitive hydrogenase of the cyanobacterial host is replaced with an O_2-tolerant hydrogenase from another bacterium. Research conducted at NREL in the past led to the discovery of an O_2-tolerant hydrogenase from the CO-oxidizing bacterium, *Rubrivivax gelatinosus* CBS (5). The CBS hydrogenase belongs to the class of NiFe-hydrogenases, which are also found in cyanobacteria. The native cyanobacterial hydrogenase, however, is extremely sensitive to O_2. Since cyanobacteria already have the complex machinery necessary to synthesize, assemble, and activate NiFe-hydrogenases, they should express the recombinant CBS enzyme as well. If successful, the proposed work could help achieve the overall goal of the project more quickly.

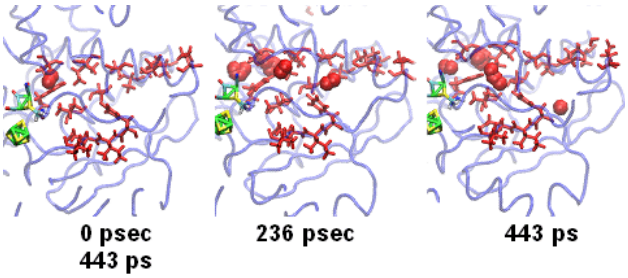

Figure 2. Computational Simulation of O_2 Gas Diffusion from the Catalytic Site to the Surface of the CpI Fe-Hydrogenase Enzyme at Three Time Points

Results

Subtask 1 (Molecular Engineering of the Algal Hydrogenase): There is a high degree of homology between the algal hydrogenase, which has not been crystallized, and the *Clostridium pasteurianum* CpI hydrogenase, which has. This homology allowed us to use the latter's solved X-ray structure to gain information about the algal enzyme. We performed molecular dynamics simulations of O_2 gas diffusion pathways through CpI in collaboration with Prof. K. Schulten's group at the Beckman Institute of the University of Illinois and NREL's Computational Sciences Center. Figure 2 shows one of the simulations, in which 10 O_2 atoms (red circles) were "loaded" next to the catalytic site of the enzyme (shown in green and yellow) at time t = 0. The motion of the O_2 atoms was simulated for a total period of 450 picoseconds. It is clear that most of the O_2 atoms diffuse through the channel, confirming our hypothesis that the channel constitutes the preferential pathway for O_2 access to the catalytic site of the enzyme. Similar simulations have identified targets for site-directed mutagenesis aimed at preventing O_2 inactivation of the homologous algal enzyme.

The implementation of the selected mutations was done *in vitro*. Our initial plans were to re-transform algal cells with the mutated genes and assay for changes in overall sensitivity of H_2-production activity to O_2. However, algal transformation is fairly inefficient (1×10^{-6}), and algal cultures grow slowly (doubling times of 8-12 hours). This limits the number of mutants that can be successfully tested over a short period of time. Nonetheless, a major accomplishment from our group this year (6), partially supported by the DOE Office of Science, allowed us to use an *E. coli* expression system to more efficiently (1×10^{-3}) and rapidly (doubling time of 20 minutes) obtain large amounts of algal hydrogenase. This was possible through the discovery of three hydrogenase assembly genes in *C. reinhardtii* that, when co-transformed with the hydrogenase structural gene in *E. coli*, result in the expression of active algal hydrogenase.

Subtask 2 (Biochemical and Process Engineering): To optimize the continuous H_2 production system, we investigated the effects of a series of experimental variables. The highest H_2 production rate (0.6 ml/h) was obtained when (a) 60 µM sulfate was introduced into the first reactor, (b) the light intensity incident on the second reactor was 50 µE m^{-2} s^{-1}, and (c) the $D_{1\text{-}2}$ dilution rate of the second reactor with cell suspension from the first reactor was 0.02 h^{-1}. Since the observed production rates were lower than the capacity of the algae to extract electrons from water, there are biological limitations unrelated to engineering factors. To address this issue, we will examine the levels of possible limiting electron transport components and alternative pathways that may compete with the hydrogenase reaction. Significantly, we extended the operation of the system to 6 months, but at variable H_2-production rates.

The development of a continuous system was based on its potential to lower the estimated cost of H_2 produced compared to the batch system (4). Concomitantly, we investigated the possibility of using immobilized, high-density algal cultures for H_2 production. Our collaborators in Russia have demonstrated that sulfur-deprived algae immobilized on fiberglass matrices photoproduce H_2 for much longer periods of time than the batch suspension system at a 1.7 times higher rate per volume of bioreactor (Laurinavichene et al., submitted). These results suggest that it may be possible to find even less expensive more flexible matrices to achieve similar increases in rates, and this will be a focus of future work.

Subtask 3 (Recombinant Cyanobacterial H_2 Production): The first step in the generation of a recombinant cyanobacterium carrying the foreign CBS hydrogenase genes requires the inactivation of

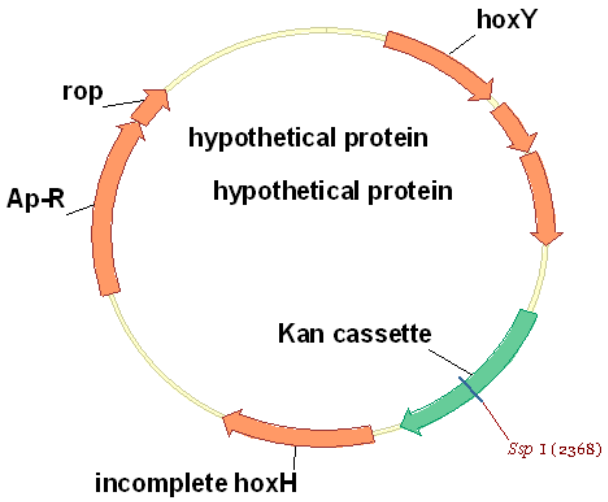

Figure 3. Plasmid (pSMART LC-Amp) Containing the *hox* amplicon with a Mutated *hoxH* (*hoxH::KanR*) Gene

the native, O_2-sensitive hydrogenase and the creation of a host hydrogenase knockout. We have thus constructed a plasmid to exchange the host *hoxH* gene with a mutated, incomplete gene (Figure 3). The plasmid will next be implemented into cyanobacteria.

The linkage between photosynthetic water oxidation and hydrogenase H_2 production depends on the activity of a mediator, ferredoxin. It is not clear, at this point, how species-specific ferredoxin is, or how well the cyanobacterial ferredoxin will interact with the CBS hydrogenase in the recombinant system. To test their interaction *in vitro*, we constructed an *E. coli* expression system to mass-produce cyanobacterial ferredoxin. The cyanobacterial ferredoxin produced by *E. coli* carries a tag and could be purified easily by affinity chromatography. The *in vitro* assay will utilize recombinant ferredoxin, photosynthetic membranes isolated from the cyanobacterium and CBS hydrogenase. In parallel with the linkage experiments, we will start the transformation studies.

Conclusions

- The discovery of an *E. coli* bacterial expression system for algal hydrogenases and the usefulness of computational simulation methods in guiding experimental site-directed mutagenesis have significantly improved our capability for generating O_2-tolerant algal hydrogenases.
- We demonstrated that both suspended and immobilized algal cultures photoproduce H_2 under sulfur deprivation in batch and continuous modes. Future optimization studies and economics analyses will help guide in the selection of the most cost-effective approach.
- A promising new approach to develop a photobiological H_2-producing organism has been initiated, based on the previous discovery of an O_2-tolerant bacterial hydrogenase.

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3. Melis A., Zhang L., Forestier M., Ghirardi M.L. and Seibert M. (2000) Plant Physiol. 122, 127-135.

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6. Posewitz M.C., Smolinski S.L., King P.W., Zhang L., Seibert M. and Ghirardi M.L. (2004) J. Biol. Chem. 279, 25711-25720.

FY 2004 Publications

1. Antal, TK, TE Krendeleva, AB Rubin, TV Laurinavichene, AA Tsygankov, VV Makarova, S Kosourov, ML Ghirardi and M Seibert. **2003**. The dependence of algal H_2 production on Photosystem II and O_2-consumption activity in sulfur-deprived *Chlamydomonas reinhardtii* cells. *Biochim. Biophys. Acta* 1607, 153-160.

2. Ghirardi, ML and W. Amos. **2004**. Hydrogen photoproduction by sulfur-deprived green algae – status of the research and potential of the system. *Biocycle* 45, 59.

3. Hahn, JJ, ML Ghirardi and WA Jacoby. **2004**. Effect of process variables on photosynthetic algal hydrogen production. *Biotechnol. Progr.* 20, 989-991.

4. Posewitz, MC, PW King, SL Smolinski, L Zhang, M Seibert and ML Ghirardi. **2004**. Discovery of two novel Radical SAM proteins required for the assembly of an [Fe]-hydrogenase. *J. Biol. Chem*. 279, 25711-25720.

5. Merida, W, PC Maness, RC Brown, and DB Levin. **2004.** Enhanced hydrogen production from indirectly heated, gasified biomass, and removal of carbon gas emissions using a novel biological gas reformer. *Intl. J. Hydrogen Energy*. 29: 283-290.

FY 2004 Presentations

1. "Algal H_2 Production—Physiology, Process Development, and Hydrogenase Molecular Engineering" by M. Seibert at the Beckman Institute, University of Illinois, Urbana, IL, May 28, 2003.

2. "Algal H_2 Production—Physiology, Process Development, and Hydrogenase Molecular Engineering" by M. Seibert, Institute for Biological Energy Alternatives, Rockville, MD, June 12, 2003.

3. "Hydrogen Production via Fermentation" by P.C. Maness, Tyndall Air Force Base, Panama City, FL, June 2003.

4. "Algal H_2 Production—An American Approach" by M. Seibert, MBD2003/IEA Annex 15 Meeting, Chiba, Japan, September 23, 2003.

5. "Molecular Engineering of an Algal Hydrogenase for Improved O_2 Tolerance" by M. Seibert, COST 841 Meeting, Mülheim, Germany, September 29, 2003.

6. Boichenko, VA, E Greenbaum and M Seibert. 2004.Hydrogen production by photosynthetic microorganisms. In Photoconversion of Solar Energy: Molecular to Global Photosynthesis (MD Archer and J Barber, eds) Vol 2, Imperial College Press, London, pp 397-452.

7. "Issues and Recent Progress in Algal Photobiological Hydrogen Production in the U.S." by M. Ghirardi at the DICP Symposium on Marine Biotechnology, Dalian, China, October 15-16, 2003.

8. "Molecular Engineering the *Chlamydomonas reinhardtii* [Fe]-Hydrogenase to Improve Its O_2 Sensitivity" by M. Ghirardi at the 5th Asia-Pacific Conference on Algal Biotechnology, Qingdao, China, October 18-21, 2003.

9. "Algal H_2 Production—Physiology and Hydrogenase Molecular Engineering" by M. Seibert at the DuPont Chesapeake Meeting, Chestertown, MD, October 27-30, 2003.

10. "Algal H_2-Production by Light-Driven Water-Splitting, Issues and Recent Progress" by M. Ghirardi at the BioCycle conference, Minneapolis, MN, November 17-19, 2003.

11. "*In situ* PAM Fluorescence Measurements of Sulfur-Deprived *Chlamydomonas reinhardtii* Cultures" by M. Ghirardi at the 13th Western Photosynthesis Conference in Pacific Grove, CA, January 2004.

12. "Hydrogen Production via Biomass Fermentation" by P.C. Maness at the University of Colorado at Boulder, Boulder, CO, February 2004.

13. M. Seibert ran the Genomics: GTL Roadmapping Workshop on new facilities for energy production (H_2-production) as a courtesy to the Office of Biological and Environmental Research (SC) and gave two presentations at the workshop, March 2004.

14. "Coupling the Water-Splitting Process to Hydrogen Production in Algae" by M. Seibert, International Symposium on Protein Cofactor Interactions in Biological Processes, Berlin, Germany, May 14, 2004.

Special Recognitions & Awards/Patents Issued

1. M. Ghirardi received NREL's Staff Award for Outstanding Performance, January 2004.

2. P.C. Maness was asked to serve on the Thesis Committee for a Master degree student from the Dept of Aerospace Engineering, University of Colorado at Boulder, and to advise with the student's thesis "Characterization of the effect of butyrate production in biophotolysis for use in Martian Resource Recovery", January 2004.

3. M. Seibert was asked by the Air Force Office of Scientific Research to act as one of four outside advisors for a new program in the Life Sciences Division that will fund basic Biohydrogen research starting in FY 2005, March 2004.

4. An International Patent Cooperation Treaty (PCT) application on "Oxygen-Tolerant Hydrogenases and Methods for Designing and Making Same" by King, Ghirardi and Seibert was further refined as a first step in obtaining intellectual property rights for the above subject, April 2004.

5. M. Ghirardi received DOE Office of Science's award for Outstanding Mentoring, June 2004.

6. M. Seibert was designated an NREL Research Fellow, October, 2003.